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(71) Applicant *(for all designated States except US):* ALPHA BETA TECHNOLOGY, INC. [US/US]; Two Biotechnology Park, 373 Plantation Street, Worcester, MA 01605 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* JAMAS, Spiros [ZM/US]; 173 Commonwealth Avenue, Boston, MA 02116 (US). EASSON, D., Davidson, Jr. [US/US]; 34 Harrington Farms, Shrewsbury, MA 01545 (US). OSTROFF, Gary, R. [US/US]; 327 Plantation Street, #2, Worcester, MA 01605 (US).

(74) Agents: BROOK, David, E. et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

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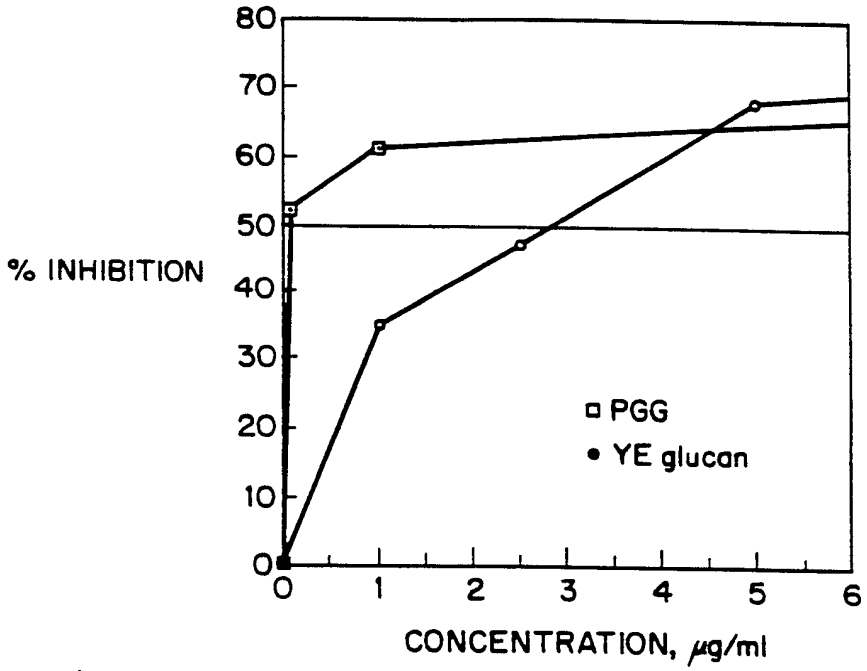

(57) Abstract

us905042 for producing soluble preparations of neutral glucan polymers is disclosed. The method involves treating whole glucan particles with a unique sequence of acid and alkaline treatments to produce soluble glucan. The soluble glucan can be purified to obtain a physiologically acceptable solution of neutral glucan molecules. A soluble neutral glucan preparation is obtained which forms a clear solution at a neutral pH and is equilibrated in a pharmaceutically acceptable carrier.

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METHOD FOR PRODUCING SOLUBLE GLUCANS

Description

Background

Glucans are generally described as polymers of glucose and are derived from yeast, bacteria, fungi and plants. Glucans containing a β(1-3)-linked glucopyranose backbone have long been known to have biological activity, specifically they have been shown to activate the immune system.

Neutral β(1-3) glucan polymers are limited in their utility in parenteral pharmaceutical applications, however, because they are not readily soluble in physiological media. DiLuzio, U.S. Patent No. 4,739,046 and Williams et al., U.S. Patent No. 4,761,402. The primary reason for the inherent insolubility of β(1-3) glucans is their tendency to form tightly associated triple-helical fibrils which resist hydration. For this reason, attempts to develop soluble β(1-3) glucans depend on chemical substitution with charged groups, such as phosphate (U.S. Patent Nos. 4,739,046; 4,761,402), amine (U.S. Patent No. 4,707,471) or other functional groups (e.g., sulphate) which change the native conformation of the glucan molecules and may affect their biological and pharmacokinetic properties.

Summary of the Invention

The present invention relates to a method for producing soluble glucan (also referred to as PGG)

preparations. In the present method, insoluble glucans are processed through a unique sequence of acid and alkaline treatments to produce soluble glucan. The soluble glucan is then purified at an alkaline pH and below a critical concentration, to obtain a soluble glucan preparation appropriate for parenteral (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), topical, oral or intranasal administration to humans and animals. Soluble glucan produced by the present method can be maintained in a clear solution when neutralized to pH 7 and equilibrated in a pharmaceutically acceptable carrier. Glucan produced by the present method is a safe, potent immune system enhancer when administered to an individual. Safe and efficacious preparations of soluble glucan polymers of the present invention can be used in therapeutic and/or prophylactic treatment regimens of humans and animals to enhance their immune response.

Brief Description of the Figures

Figure 1 is a graph showing the dose-dependent inhibitory effect on monocyte ingestion of Zymosan by soluble, modified glucan derived from S. cerevisiae R4 compared to yeast extract (YE) glucan.

Figure 2 is a graph showing the change in peripheral total and differential white blood cell (WBC) counts in mice after a single, intravenous dose of PGG (5 mg/mouse).

Figure 3 is a graph showing peripheral total and differential white blood cell (WBC) counts in mice after multiple dose sub-cutaneous

administration of PGG (5 mg/mouse/day x 4 days).

Figure 4 is a graph showing the efficacy of the PGG glucans in an E. coli sepsis model in mice.

Detailed Description of Invention

The soluble glucan preparations of this invention are prepared from insoluble glucan particles. Soluble glucan is also referred to herein as PGG (poly-(1-6)-β-D-glucopyranosyl-(1-3)-β-D-glucopyranose). Preferably, insoluble glucans derived from yeast organisms are employed. Manners et al., Biol. J., 135:19-30, (1973). Glucan particles which are particularly useful as starting materials in the present invention are whole glucan particles described by Jamas et al., in U.S. Patent No. 4,810,646, and in co-pending U.S. applications Serial Nos. 07/297,982 and 07/297,752 by Jamas et al., filed January 17, 1989, and by Jamas et al. in co-pending U.S. application Serial No. 07/333,630 filed April 5, 1989, the teachings of all of which are hereby incorporated herein by reference. The source of the whole glucan particles can be the broad spectrum of glucan-containing fungal organisms which contain β-glucans in their cell walls. Whole glucan particles obtained from the strain Saccharomyces cerevisiae R4 (NRRL Y-15903) described by Jamas et al. in co-pending application 07/333,630 are particularly useful. The structurally modified glucans hereinafter referred to as "modified glucans" derived from S. cerevisiae R4 are potent immune system activators, as described in co-pending

U.S. Application Serial No. 07/404,765, filed September 8, 1989, by S. Jamas, D.D. Easson, Jr. and G. Ostroff, (Attorney's Docket No. ABY89-01), the teachings of which are hereby incorporated herein by reference.

The whole glucan particles utilized in this present invention can be in the form of a dried powder, as described by Jamas et al., in U.S. Patent No. 4,810,646 and in co-pending applications USSN 07/297,982, 07/297,752 and 07/333,630. For the purpose of this present invention it is not necessary to conduct the final organic extraction and wash steps described by Jamas et al.

In the present process, whole glucan particles are suspended in an acid solution under conditions sufficient to dissolve the acid-soluble glucan portion. For most glucans, an acid solution having a pH of from about 1 to about 5 and at a temperature of from about 20 to about 100°C is sufficient. Preferably, the acid used is an organic acid capable of dissolving the acid-soluble glucan portion. Acetic acid, at concentrations of from about 0.1 to about 5M or formic acid at concentrations of from about 50% to 98% (w/v) are useful for this purpose. The treatment time may vary from about 10 minutes to about 20 hours depending on the acid concentration, temperature and source of whole glucan particles. For example, modified glucans having more β(1-6) branching than naturally-occurring, or wild-type glucans, require more stringent conditions, i.e., longer exposure times and higher temperatures. This acid-treatment step can be repeated under similar or

variable conditions. In one embodiment of the present method, modified whole glucan particles from the strain, S. cerevisiae R4, which have a higher level of β(1-6) branching than naturally-occuring glucans, are used, and treatment is carried out with 90% (by wt.) formic acid at 20°C for about 20 minutes and then at 85°C for about 30 minutes.

The acid-insoluble glucan particles are then separated from the solution by an appropriate separation technique, for example, by centrifugation or filtration. The pH of the resulting slurry is adjusted with an alkaline compound such as sodium hydroxide, to a pH of about 7 to about 14. The slurry is then resuspended in hot alkali having a concentration and temperature sufficient to solubilize the glucan polymers. Alkaline compounds which can be used in this step include alkali-metal or alkali-earth metal hydroxides, such as sodium hydroxide or potassium hydroxide, having a concentration of from about 0.1 to about 10 N. This step can be conducted at a temperature of from about 4°C to about 121°C, preferably from about 20°C to about 100°C. In one embodiment of the process, the conditions utilized are a 1 N solution of sodium hydroxide at a temperature of about 80-100°C and a contact time of approximately 1-2 hours. The resulting mixture contains solubilized glucan molecules and particulate glucan residue and generally has a dark brown color due to oxidation of contaminating proteins and sugars. The particulate residue is removed from the mixture by an

appropriate separation technique, e.g., centrifugation and/or filtration. In another embodiment of the process the acid-soluble glucans are precipitated after the preceding acid hydrolysis reaction by the addition of about 1.5 volumes of ethanol. The mixture is chilled to about 4°C for two (2) hours and the resulting precipitate is collected by centrifugation or filtration and washed with water. The pellet is then resuspended in water, and stirred for three (3) to twelve (12) hours at a temperature between about 20°C and 100°C. At this point the pH is adjusted to approximately 10 to 13 with a base such as sodium hydroxide.

The resulting solution contains soluble glucan molecules. This solution can, optionally, be concentrated to effect a 5 to 10 fold concentration of the retentate soluble glucan fraction to obtain a soluble glucan concentration in the range of about 1 to 10 mg/ml. This step can be carried out by an appropriate concentration technique, for example, by ultrafiltration, utilizing membranes with nominal molecular weight levels (NMWL) or cut-offs in the range of about 1,000 to 100,000 daltons. It was discovered that in order to prevent gradual aggregation or precipitation of the glucan polymers the preferred membrane for this step has a nominal molecular weight cut-off of about 100,000 daltons.

The concentrated fraction obtained after this step is enriched in the soluble, biologically active glucan PGG. To obtain a pharmacologically acceptable solution, the glucan concentrate is further purified, for example, by diafiltration

using a 10,000 dalton membrane. In one embodiment of the present method, diafiltration is carried out using approximately 10 volumes of alkali in the pH range of about 11 to 13. The preferred concentration of the soluble glucan after this step is from about 2 to about 10 mg/ml. The pH of the solution is adjusted in the range of about 7-12 with an acid, such as hydrochloric acid. Traces of proteinaceous and lipid materials which may be present can be removed by contacting the resulting solution with a positively charged medium such as DEAE-cellulose, QAE-cellulose, Q-Sepharose or hydrophobic interaction resins. Proteinaceous material is detrimental to the quality of the glucan product, may produce a discoloration of the solution and aids in the formation of gel networks, thus limiting the solubility of the glucan polymers. A clear solution is obtained after this step, which is neutralized to pH 7 with hydrochloric acid.

The highly purified, clear glucan solution can be further purified, for example, by diafiltration, using a pharmaceutically acceptable medium (e.g., sterile water for injection, phosphate-buffered saline (PBS), isotonic saline, dextrose) suitable for parenteral administration. The preferred membrane for this diafiltraton step has a nominal molecular weight cut-off of about 10,000 daltons. The final concentration of the glucan solution is adjusted in the range of about 0.5 to 10 mg/ml. In accordance with pharmaceutical manufacturing standards for parenteral products, the solution can

be terminally sterilized by filtration through a 0.22 μm filter. The soluble glucan preparation obtained by this process is sterile, non-antigenic, and essentially pyrogen-free, and can be stored at room temperature for extended periods of time without degradation. This process is unique in that it results in a neutral aqueous solution of immunologically active glucans which is suitable for parenteral administration and which meets the following specifications:

Endotoxin	<3.0 EU/mg
Bioburden	0 CFU/ml
Glucose	>98% (by weight)
Protein	<0.5% (by weight)
Glycogen	<0.5% (by weight)
Chitin	<0.5% (by weight)
Lipid	<0.1% (by weight).

For purposes of the present invention, the term "soluble" as used herein to describe glucans obtained by the present process, means a visually clear solution can be formed in an aqueous medium such as water, PBS, isotonic saline, or a dextrose solution having a neutral pH (e.g., about pH 5 to about 7.5), at room temperature (about 20-25°C) and at a concentration of up to about 10 mg/ml. The term "aqueous medium" refers to water and water-rich phases, particularly to pharmaceutically acceptable aqueous liquids, including PBS, saline and dextrose solutions.

A critical advantage of this method is that drying or reconstitution of the soluble glucan polymer is not required at any point in the process.

The resulting solution is substantially free of protein contamination, is non-antigenic, non-pyrogenic and is pharmaceutically acceptable for parenteral administration to animals and humans. However, if desired, the soluble glucan can be dried by an appropriate drying method, such as lyophilization, and stored in dry form. The dried glucan can be reconstituted prior to use by adding an alkali solution such as about 0.1-0.4N NaOH and reprocessed starting from the step immediately following the organic acid contact steps described above.

The soluble glucans produced by the method of this invention are branched polymers of glucose, referred to as PGG, containing β(1-3) and β(1-6) linkages in varying ratios depending on the organism and processing conditions employed. Preferably, PGG is produced from Saccharomyces cerevisiae R4, which results in a high β(1-6)/β(1-3) ratio. These glucans have shown superior immunological properties, as described in co-pending U.S. patent application serial no. 07/404,765, referenced above. The PGG glucan preparations contain glucans, which have not been substantially modified by substitution with functional (e.g., charged) groups or other covalent attachments. The biological activity of PGG glucan can be controlled by varying the average molecular weight and the ratio of β(1-6) to β(1-3) linkages of the glucan molecules, as described by Jamas et al. in U.S. Patent 4,810,646 and in co-pending applications USSN 07/297,982, 07/297,752

and 07/333,630. The average molecular weight of soluble glucans produced by the present method is generally from about 10,000 to about 500,000 daltons, preferably from about 30,000 to about 50,000.

The present soluble glucan preparations can be used as safe, effective, therapeutic and/or prophylactic agents, either alone or as adjuvants, to enhance the immune response in humans and animals. Soluble glucans produced by the present method enhance or prime the immune system so that the immune response is quicker and more pronounced. The present soluble glucan composition can be used to prevent or treat infectious diseases in malnourished patients, patients undergoing surgery, patients undergoing chemotherapy or radiotherapy, neutropenic patients, HIV-infected patients, trauma patients, burn patients and the elderly, all of whom may have weakened immune systems. Methods of treating immunocompromised patients with glucans are described in detail in co-pending U.S. application Serial No. 07/404,765 by Jamas et al., referenced above.

The present composition is generally administered to an animal or a human in an amount sufficient to produce immune system enhancement. The preparation can be administered parenterally by injection, e.g., subcutaneously, intravenously, intramuscularly, intraperitoneally, subcutaneously, topically, orally or intranasaly. The soluble glucans can be administered as a clear solution having a concentration of from about 1 mg/ml to

about 10 mg/ml. The solvent can be a physiologically acceptable aqueous medium, such as water, saline, PBS or a 5% dextrose solution. The amount necessary to induce immune system enhancement will vary on an individual basis and be based at least in part on consideration of the individual's size, the severity of the symptoms and the results sought.

PGG is a non-toxic, non-antigenic glucan preparation which enhances or primes the body's natural defense against infection, particularly for patients with normal or decreased immunologic function, so that the normal immune response is faster and more pronounced. Parenteral administration of PGG mimics the natural physiologic response to an infectious challenge by enhancing the balanced, endogenous release of cytokines in appropriate quantities and proportions. PGG can be used for the prevention and treatment of infections caused by a broad spectrum of bacterial, fungal, viral and protozoan pathogens. The prophylactic administration of PGG to a person undergoing surgery, either preoperatively, intraoperatively and/or post-operatively, will reduce the incidence and severity of post-operative infections in both normal and high-risk patients. For example, in patients undergoing surgical procedures that are classified as contaminated or potentially contaminated (e.g., gastrointestinal surgery, hysterectomy, cesarean section, transurethal prostatectomy) and in patients in whom infection at

the operative site would present a serious risk (e.g., prosthetic arthroplasty, cardiovascular surgery), concurrent initial therapy with an appropriate antibacterial agent and the present PGG preparation will reduce the incidence and severity of infectious complications.

In patients who are immunosuppressed, not only by disease (e.g., cancer, AIDS) but by courses of chemotherapy and/or radiotherapy, the prophylactic administration of PGG will reduce the incidence of infections caused by a broad spectrum of opportunistic pathogens including many unusual bacteria, fungi and viruses. Therapy using PGG has demonstrated a significant radioprotective effect with its ability to enhance and prolong macrophage function and regeneration and, as a result enhance resistance to microbial invasion and infection.

In high risk patients (e.g., over age 65, diabetics, patients having cancer, malnutrition, renal disease, emphysema, dehydration, restricted mobility, etc.) hospitalization frequently is associated with a high incidence of serious nosocomial infection. Treatment with PGG glucan may be started empirically before catheterization, use of respirators, drainage tubes, intensive care units, prolonged hospitalizations, etc. to help prevent the infections that are commonly associated with these procedures. Concurrent therapy with antimicrobial agents and the PGG is indicated for the treatment of chronic, severe, refractory, complex and difficult to treat infections.

Glucan produced by the present method enhances the non-specific defenses of mammalian mononuclear cells and significantly increases their ability to respond to an infectious challenge. The unique property of glucan-macrophage activation is that it does not result in increased body temperatures (i.e., fever) as has been reported with many non-specific stimulants of host defenses. This critical advantage of glucan may lie in the natural profile of responses it mediates in white blood cells. A unique mechanism of the soluble PGG glucan of the present invention is that pre-treatment of normal human leukocytes with PGG in vitro appears to prime the mononuclear cells to release elevated levels of monokines (TNF, GM-CSF, M-CSF, IL-1, IL-6) only upon subsequent stimulation with endotoxin or other infectious agents. The soluble PGG glucan of the present invention is therefore unique from other glucan preparations (e.g., lentinan, kreshin) and immunostimulants in that it does not directly stimulate IL-1 and TNF release from mononuclear cells. This is considered highly advantageous since the monokines are not released systemically until exposure to the infectious agent. Thus, the present invention provides a soluble glucan which can be parenterally, topically, intranasaly, or orally administered to an animal or human to enhance the immune system, and a method for producing the soluble glucan.

The invention is further illustrated by the following Examples.

EXAMPLES

Example 1

Preparation of PGG from Dried Whole Glucan Particles

Whole glucan particles were prepared from dried Baker's Yeast (Universal Foods, WI) according to the procedure of Jamas et al., U.S. Patent No. 4,810,646. 100 grams of the resulting dried whole glucan particles were resuspended in 3 liters of 90% formic acid and stirred at room temperature for 1 hour. The mixture was then heated to 80°C and stirred until a sudden drop in viscosity was observed. At this point, 9 liters of ethanol were added to the mixture resulting in formation of a precipitate, which was collected by centrifugation. The precipitate was then dissolved in 0.4 M sodium hydroxide (NaOH) and the solution was centrifuged to remove undissolved particulates. The supernatant was concentrated by ultrafiltration using an Immersible-Cx-30 Ultrafilter (Millipore Corp., Bedford, MA) with a 30,000 dalton nominal molecular weight limit (NMWL) cut off. The retentate fraction was then diafiltered with ten volumes of water using the same equipment. The resulting solution was concentrated and equilibrated in sterile isotonic saline by diafiltration. The final yield of this fraction (>30,000 daltons) was 1.9 grams.

To produce a 10,000-30,000 fraction, the filtrate from the first ultrafiltration was concentrated by ultrafiltration through a 10,000

dalton membrane using an Immersible-CX-10 Ultrafilter (Millipore Corp.). The concentrated retentate fraction was then diafiltered with ten volumes of water, followed by equilibration in sterile isotonic saline. The final yield of this fraction was 2.7 grams.

Example 2

Production of PGG from Saccharomyces cerevisiae R4

Saccharomyces cerevisiae R4 (NRRL Y-15903) was cultured in 60 liters of a defined growth medium (4.45 g/l KH_2PO_4, 3.0 g/L $(NH_4)_2SO_4$, 1.1 g/l $MgSO_4.7H_2O$, 1.8 g/l Lysine-HCl, 0.9 g/l Tyrosine, 0.012 g/l Adenine, 0.012 g/l Uracil, 5.0 g/l casamino acids, 0.45 g/l Histidine and 4.0 g/l Glucose) in a MPP-80 Mobile Pilot Plant Fermenter (New Brunswick Scientific, NJ). When the culture reached an optical density (OD, 600nm) of 30 the fermentation was stopped by adjusting the pH to 12 with 5M sodium hydroxide. The total cell yield was approximately 1.8 kg dry cell weight. The cells were harvested by centrifugation using a Westfalia Nozzle Bowl Separator (Model SKOG-205, Centrico, NJ) and were washed with water. The concentrated cell suspension was transferred to a stainless steel stirred vessel and resuspended in 10 liters of 1M sodium hydroxide and stirred for 20 hours at 25°C. The mixture was then heated to 90°C and stirred for an additional 1 hour. The insoluble particles were collected by centrifugation and washed with water.

The concentrated slurry was resuspended to a volume of 10 liters in 1M sodium hydroxide and stirred at 90°C for 3 hours. This extraction step was repeated at 90°C for 1 hour. The insolubles were collected by centrifugation and washed with water. The concentrated slurry was then resuspended in 10 liters of water, the pH was adjusted to 4.5 with hydrochloric acid and stirred at 90°C for 1 hour, followed by centrifugation and washing. The concentrated slurry was then resuspended in 5 liters of 0.5 M acetic acid and stirred at 90°C for 3 hours. The insolubles were collected by centrifugation. The yield of glucan particles at this step was 2.8 kg net weight.

An aliquot of 100 grams of the insoluble glucan particles was then resuspended in 500 ml of 0.5 M acetic acid and was extracted at 90°C for 20 hours. The suspension was then neutralized to pH 7 with sodium hydroxide, and the insoluble glucan particles were collected by centrifugation. The glucans were resuspended in 200 ml of 1M sodium hydroxide and heated to 90°C for 1 hour to solubilize the glucan. The mixture was cooled and centrifuged to remove particulate debris. The supernatent solution was diluted to 0.4 M sodium hydroxide with water and was filtered through a 0.5 μm polypropylene depth filter. The resulting solution was concentrated four-fold by ultrafiltration through a 10,000 dalton NMWL membrane using a Minitan HRTF System (Millipore Corp.).

The retentate fraction was then diafiltered with ten volumes of 0.4 M sodium hydroxide using the

same equipment. The solution was diluted to obtain a 2 mg/ml glucan solution in 0.225 M sodium hydroxide. The solution was adjusted to pH 9 with hydrochloric acid and diafiltered against sterile, isotonic saline using the Minitan System. The solution was then filtered through a 0.22 μm sterilizing filter. This procedure gave 1.1 grams of sterile PGG glucan with a weight average molecular weight of 225,000 daltons.

Example 3

Affinity of Modified Glucans for the Monocyte β-glucan Receptor

The ability of glucan molecules to be recognized and bound to the β-glucan receptor of monocytes is critical for their biological activity. Modified whole glucans derived from the mutant strain R4 (WGP-R4) demonstrated an increased affinity for the glucan receptor of monocytes when compare to naturally occurring glucans from Baker's yeast. Janusz et al., J. of Immunol., 137:3270-3276 (1986).

Water-soluble modified glucan (PGG) was prepared from WGP-R4 according to the procedure outlined in Example 2.

Human monocytes were incubated with various concentrations of the PGG for 15 minutes, washed to remove unbound glucan and then incubated with Zymosan for 30 minutes. After fixing and staining the monolayers, the percentage of monocytes ingesting Zymosan was determined. The affinity of

glucan preparations for the β-glucan receptor by was measured according to their ability to competitively occupy the receptor thus inhibiting the uptake of Zymosan by monocytes. Samples were compared by taking the concentration of glucan required to obtain 50% inhibition of Zymosan ingestion.

The significantly enhanced affinity of the soluble PGG glucan derived from WGP-R4 to the receptor is evident by the low concentration required to obtain a 50% inhibition of Zymosan ingestion. The results, presented in Figure 1, demonstrate that the PGG glucan, designated WGP-R4, binds to the monocyte β-glucan receptor with a much higher affinity (0.1 μg/ml) than soluble glucan from Baker's yeast extract (3.5 μg/ml), (YE glucan) representing a 35-fold increase in activity.

Example 4

Effect of PGG Molecular Weight on Macrophage Phagocytosis

Two molecular weight fractions of PGG from Saccharomyces cerevisiae R4 were prepared according to the procedure outlined in Example 2. The PGG preparations were then assayed for their affinity to the monocyte β-glucan receptor by measuring inhibition of Zymosan phagocytosis as described in Example 3. The results, shown in Table 1 demonstrate that the molecular weight of the PGG preparations affects their affinity for the β-glucan receptor, and therefore is expected to affect their in vivo immunologic activity.

TABLE 1

Effect of PGG Molecular Weight on Receptor Binding

Glucan	Concentration for 50% Inhibition μg/ml	Relative Avidity
Barley β-Glucan[1]	65	1
PGG-R4 Modified Glucan MW = 20,000d	0.6	108
PGG-R4 Modified Glucan MW = 330,000	0.1	650

[1] Czop and Austen., J. Immunology, 135(5):3388-3393, (1985).

Example 5

In Vivo Activity of PGG Glucans

The effect of in vivo administration of modified glucans on peripheral white blood cell (WBC) counts was characterized in mice. PGG preparations of the modified glucan from strain R4 were prepared according to the procedure outlined in Example 2 and administered intravenously (IV) and subcutaneously (SC) to male CD-1 mice. Total and differential cell counts were monitored at regular time intervals.

A profound increase in the total WBC count was observed particularly following single-dose IV administration of PGG. Figures 2 and 3 summarize

the results, which show rapid (<6 hours) amplification of total WBC counts with, the most pronounced increase (12X and 6X) occurring in the monocyte and granulocyte counts, respectively. This is consistent with in vitro data suggesting the presence of a high affinity β-glucan receptor present on human monocytes. The multiple-dose SC regimen (Figure 3) elicited an increase in total WBC beginning at 48 hours and peaking at 144 hours after initiation of therapy. The increase in total counts was consistent with an increase in the peripheral monocyte population over this time period. The average monocyte count increased from 320/mm^3 at zero hours to approximately 8,000/mm^3 at 144 hours, representing at 24-fold increase.

Example 6

Infection Model

A sepsis model was developed in mice to characterize the efficacy of modified PGG glucans in protecting an immunologically intact host against serious infections, such as those which commonly occur following abdominal surgery. PGG derived from WGP-R4 was prepared according to the procedure outlined in Example 2.

The model used intraperitoneal challenge of mice with an 0.1 ml suspension of E. coli strain TVDL-rat (approximately 10^8 CFU/ml) 24 hours following IV administration of PGG by single bolus injection using transthoracic cardiac puncture. Mice were returned to their cages and maintained on

food and water ad libitum. A control group of 10 mice were injected with 0.1 ml sterile saline at the time of the PGG administration. Mortality rates for the treatment groups and saline control group were recorded at 48 hours after challenge. The results, shown in Figure 4, demonstrated that PGG obtained from the modified glucan, WGP-R4, significantly reduced mortality, as compared to the saline control group ($p<0.05$) at doses as low as 0.01 mg/mouse (0.5 mg/kg body weight).

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

CLAIMS

1. A process for producing soluble glucan comprising the steps of:
   a. contacting glucan particles with an acid solution;
   b. contacting the acid-treated particles of step (a) with an alkali solution under conditions sufficient to dissolve alkali-soluble glucan; and
   c. separating alkali-insoluble particulates and glucan aggregates from the solution of step (b); and
   d. neutralizing the glucan solution obtained from step (c).

2. A process of Claim 1 wherein the glucan particles are whole glucan particles derived from yeast.

3. A process of Claim 2 wherein the yeast comprises a strain of S. cerevisiae.

4. A process of Claim 3 wherein the strain of S. cerevisiae is strain R4 (NRRL Y-15903).

5. A process of Claim 1 wherein the acid solution of step (a) is a solution of an organic acid.

6. A process of Claim 5 wherein the organic acid is acetic acid at a concentration of from about 0.1 to about 5 M.

7. A process of Claim 5 wherein the organic acid is formic acid at a concentration of from about 50% to 98% (w/v).

8. A process of Claim 1 wherein step (a) is performed at a temperature of about 20°C to about 100°C and for a period of about 20 minutes to about 20 hours.

9. A process of Claim 1 wherein the alkali solution of step (b) is a solution of an alkali-metal or alkali-earth metal hydroxide having a concentration of from about 0.01 to about 10.0 N and a pH of from about 7 to about 14.

10. A process of Claim 9 wherein the alkali solution is 0.1N sodium hydroxide.

11. A process of Claim 1 wherein step (b) is performed at a temperature of from about 4°C to about 121°C and for a period of about 1 to 3 hours.

12. A process of Claim 1 comprising the additional step of contacting the solution obtained after step (b) with a positively-charged medium selected from the group consisting of DEAE-cellulose, QAE-cellulose and Q-Sepharose.

13. A process of Claim 1 comprising the additional step of contacting the solution obtained after step (b) with a hydrophobic interaction medium.

14. A process of Claim 1 wherein step (c) is performed to remove particulate and aggregated glucans.

15. A process of Claim 1 wherein step (c) is performed by ultrafiltration at an alkaline pH using a membrane having a molecular weight cut-off of about 100,000 daltons.

16. Soluble glucan produced by the process of Claim 1, having a molecular weight greater than 10,000 daltons which is soluble in aqueous media, and which is non-antigenic and non-pyrogenic.

17. Soluble glucan of Claim 16 containing greater than 98% by weight glucose, less than 0.5% by weight protein, glycogen and chitin, and less than 0.1% by weight lipid.

18. A process for producing purified soluble glucan for parenteral administration to an animal or human, comprising the steps of:

a. contacting whole glucan particles with an acid solution;

b. contacting the acid-treated particles of step (a) with an alkali solution under

conditions sufficient to dissolve alkali-soluble glucan;

c. separating alkali-insoluble particulates and glucan aggregates from the solution of step (b);

d. neutralizing the solution of step (c); and

e. further purifying the solution of step (d) by diafiltration with a pharmaceutically acceptable medium to produce a purified, neutral glucan solution.

19. A process of Claim 18 wherein the whole glucan particles are derived from yeast.

20. A process of Claim 19 wherein the yeast comprises a strain of S. cerevisiae.

21. A process of Claim 20 wherein the strain of S. cerevisiae is strain R4 (NRRL Y-15903).

22. A process of Claim 16 wherein the acid solution of step (a) is a solution of an organic acid.

23. A process of Claim 22 wherein the organic acid is acetic acid or formic acid.

24. A process of Claim 18 wherein step (a) is performed at a temperature of about 20°C to about 100°C for a period of about 20 minutes to about 20 hours.

25. A process of Claim 18 wherein the alkali solution of step (c) is a solution of an alkali-metal or alkali earth-metal hydroxide having a concentration of from about 0.01 to about 10.0 N and a pH of about 7 to about 14.

26. A process of Claim 25 wherein the alkali solution is 1N sodium hydroxide.

27. A process of Claim 18 wherein step (b) is performed at a temperature of from about 4°C to about 121°C and for a period of about 1 to 3 hours.

28. A process of Claim 18 wherein step (c) is performed by diafiltration with an alkali solution having a pH of about 11 to about 14.

29. A process of Claim 18 wherein step (e) is performed by diafiltration with a pharmaceutically acceptable medium.

30. A process of Claim 29 wherein the pharmaceutically acceptable medium is selected from the group consisting of: water, PBS, isotonic saline and dextrose.

31. A process of Claim 18 further comprising contacting the solution obtained after step (b) with DEAE-cellulose, QAE-cellulose, Q-Sepharose or a hydrophobic interaction medium.

32. Soluble glucan produced by the method of Claim 18.

33. Soluble glucan of Claim 32 containing greater than 98% by weight glucose, less than 0.5% by weight protein, glycogen and chitin, and less than 0.1% by weight lipid.

34. A solution of soluble glucan produced by the method of Claim 18.

35. A solution of Claim 34 containing from about 0.5 to about 10 mg/ml glucan in a pharmaceutically acceptable medium.

36. A solution of Claim 35 wherein the pharmaceutically acceptable medium is selected from the group consisting of: water, PBS, isotonic saline and dextrose.

37. Aqueous-soluble non-derivatized glucan having an average molecular weight of from about 10,000 to about 500,000 daltons.

38. Aqueous-soluble non-derivatized glucan of Claim 37 which is derived from S. cerevisiae R4 (NRRL Y-15903).

39. Aqueous-soluble non-derivatized glucan of Claim 38 having an average molecular weight of about 30,000 to about 200,000 daltons.

40. Aqueous-soluble non-derivatized glucan of Claim 39 containing at least 98% by weight glucose, less than 0.5% by weight protein, glycogen and chitin, and less than 0.1% by weight lipid.

41. A solution for parenteral administration to a human or an animal comprising an aqueous-soluble non-derivatized glucan having an average molecular weight of about 10,000 to about 500,000 daltons in a pharmaceutically acceptable medium.

42. A solution of Claim 41 having a concentration of said glucan of from about 0.5 to about 10.0 mg/ml.

43. A solution of Claim 42 wherein the average molecular weight of said glucan is from about 30,000 to about 200,000 daltons.

44. A solution of Claim 42 wherein the pharmaceutically acceptable medium is selected from the group consisting of: water, PBS, isotonic saline and dextrose.

45. A method of stimulating an immune response in an animal or human comprising administering to said animal or human an amount of an aqueous-soluble glucan of Claim 37, sufficient to stimulate an immune response in said animal or human.

46. A method of treating an immunocompromised human or animal comprising administering to said human or animal an amount of an aqueous-soluble glucan of Claim 38 sufficient to stimulate an immune response in said animal or human.

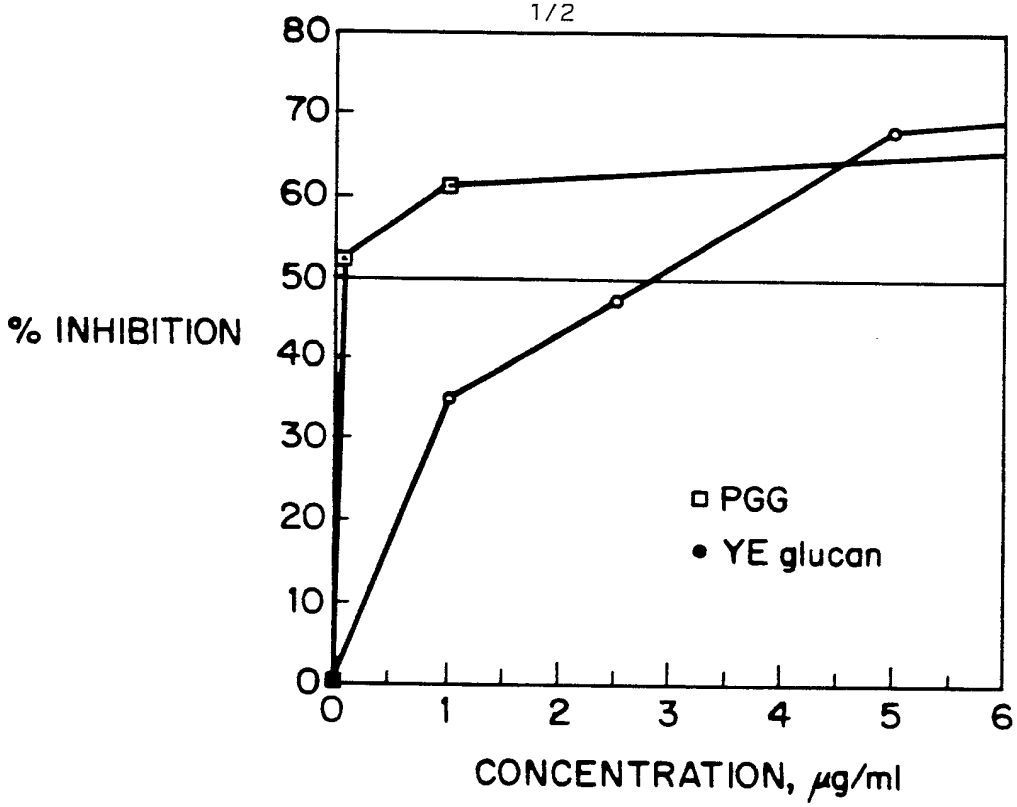
% INHIBITION
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
PGG
YE glucan
CONCENTRATION, μg/ml

Fig. 1

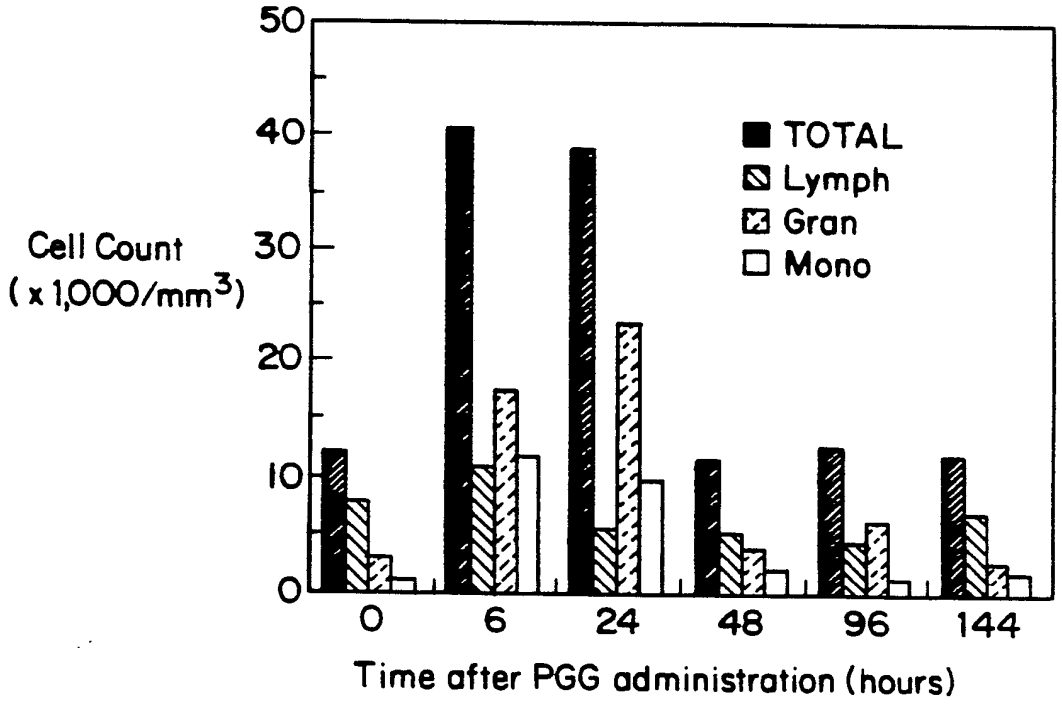
Cell Count
(x 1,000/mm3)
50
40
30
20
10
0
TOTAL
Lymph
Gran
Mono
0
6
24
48
96
144
Time after PGG administration (hours)

Fig. 2

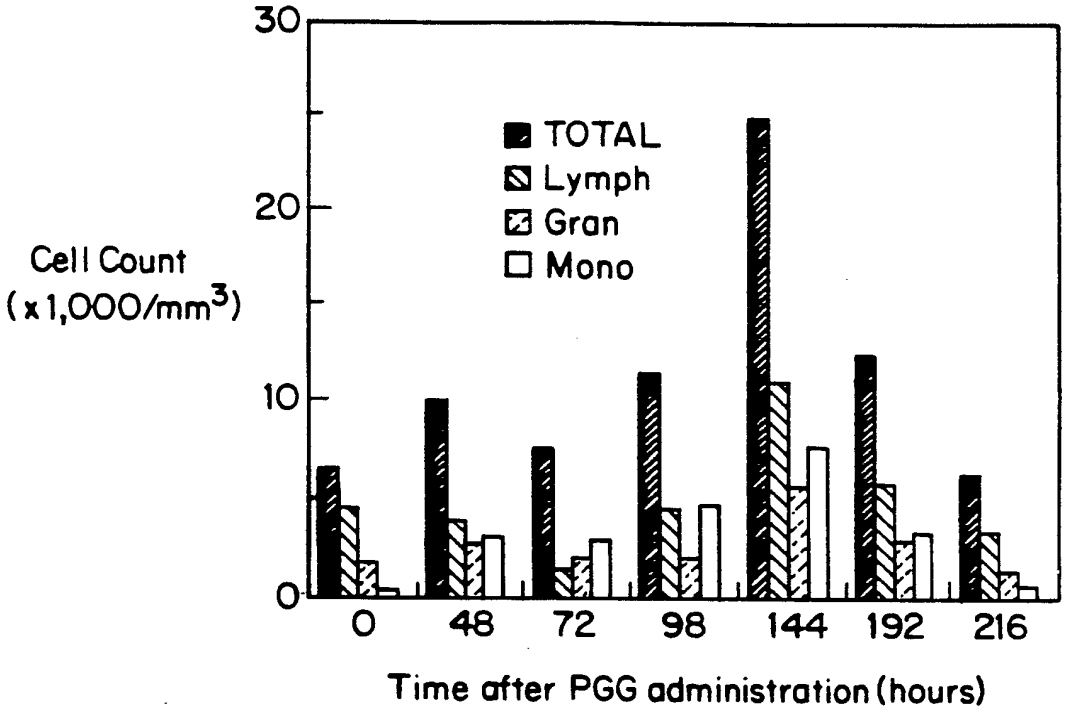
30
20
10
0
TOTAL
Lymph
Gran
Mono
Cell Count
(x1,000/mm3)
0
48
72
98
144
192
216
Time after PGG administration (hours)

Fig. 3

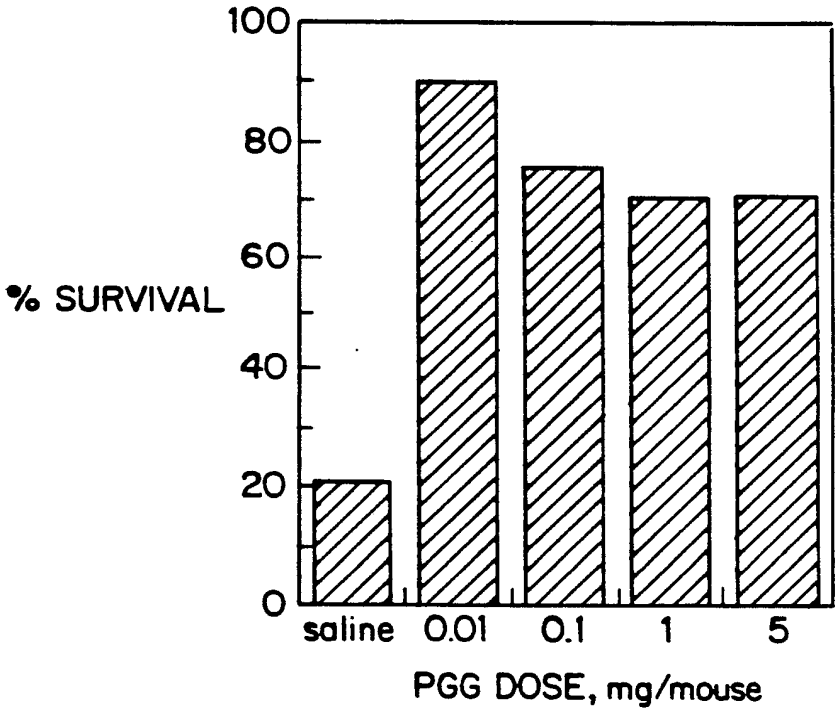
100
80
60
40
20
0
% SURVIVAL
saline
0.01
0.1
1
5
PGG DOSE, mg/mouse

Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/05041

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 08 B 37/00, A 61 K 31/715//C 12 P 19/04

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 08 B; A 61 K; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Chemical Abstracts, volume 71, no. 23, 8 December 1969, (Columbus, Ohio, US), Bacon, John S. D. et al.: "Glucan components of the cell wall of bakers' yeast (Saccharomyces cerevisiae) considered in reation to its ultrastructure ", see page 19, abstract 109168c, & Biochem. J. 1969, 114(3), 557- 567	1-5
Y		18-22
	--	
Y	Journal of Immunology, Vol. 137, No. 10, November 1986, Michael J. Janusz, et al.: "Isolation of soluble yeast beta-glucans that inhibit human monocyte phagocytosis mediated by beta-glucan receptors1 ", see page 3270 - page 3276 page 3275, column 1, 2nd paragraph	1-5,18-22
	--	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th December 1990	30. 01. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Nuria TORIBIO

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Biochem. J., Vol. 135, 1973, David J. Manners et al.: "The Structure of a beta-(1-3)-D-Glucan from Yeast Cell Walls ", see page 19 - page 30 see esp. scheme 2 and 3	1-5,18-22
Y	US, A, 4810646 (SPIROS JAMES ET AL.) 7 March 1989, abstract, examples 2 and 3	1-5
A	Chemical Abstracts, volume 85, no., 27 September 1976, (Columbus, Ohio, US), Fleet, G.H. et al.: "Isolation and composition of an alkali-soluble glucan from the cell walls of Saccharomyces cerevisiae ", see page 267, abstract 89819z, & J. Gen. Microbiol. 1976, 94(1), 180- 192	1-44
A	Chemical Abstracts, volume 109, no., 29 August 1988, (Columbus, Ohio, US), Williams, David L. et al.: "Pre-clinical safety evaluation of soluble glucan ", see page 35, abstract 66566q, & Int. J. Immunopharmacol. 1988, 10(4), 405-414	1-44

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 45,46, because they relate to subject matter not required to be searched by this Authority, namely:

Methods for treatment of the human or animal body by therapy (PCT rule 39 (iv).

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional seach fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 90/05041

SA 40523

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 28/11/90
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4810646	07/03/89	NONE	

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

EPO FORM P0479